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14. ABSTRACT: The purpose of this research was to develop a gene delivery system that can target breast cancer cells specifically and transfect them efficiently. Using component integration approach, several subsystems of diverse biological origin were integrated onto a modular platform in order to carry diverse functions of an efficient gene delivery vehicle. A biomimetic vector was genetically engineered to contain at precise locations: a) an adenovirus μ peptide to condense pDNA into nanosize particles, b) a combinatorially-screened synthetic peptide to target breast cancer cells and enhance internalization of nanoparticles, c) a pH-responsive synthetic fusogenic peptide to disrupt endosome membranes and facilitate escape of the nanoparticles into the cytosol, and d) a nuclear localization signal from human immuno-deficiency virus for microtubule mediated transfer of genetic material to the nucleus. The vector was characterized physicochemically and biologically and the results demonstrated that a fully functional vector can be engineered to target breast cancer cells with high specificity, mimic virus characteristics and overcome the biological barriers associated with targeted gene transfer.

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Introduction

The **overall purpose** of this project was to develop a new generation of programmable non-viral vectors to target and transfect breast cancer cells efficiently. **The rationale** is that a multidomain designer vector based on a One-Domain-One-Function architecture can effectively perform an array of functions including a) **self assembly** into vector/DNA nanoparticles i.e., packaging the therapeutic gene into right sized packets, b) **self-guidance**, i.e. identification and targeting the breast cancer cells for specificity, c) **self-preservation**, i.e., escaping the endosomal compartment and nucleases inside the cells and, d) **assured-delivery**, i.e., successful localization into the nucleus for the expression of therapeutic gene. Two Specific Aims were proposed:

a) Synthesize four designer vectors (DCM, NLS-DCM, NLS-DCM-EDM, and NLS-DCM-EDM-CCS-TP),

b) Complex the vectors with pDNA to form nanoparticles and characterize the complexes using physicochemical and biological assays.

Body

For months 1-12 the following tasks were proposed. All the dead lines were met in time.

Task 1- Synthesize/Biosynthesize DCM, NLS-DCM, NLS-DCM-EDM and NLS-DCM-EDM-CCS-TP (months 0-6).

To perform the task:

1.1. The amino acid sequences of DCM and NLS-DCM were custom synthesized using solid phase peptide synthesis by Anaspec Inc. (San Jose, CA).

1.2. The gene encoding NLS-DCM-EDM and NLS-DCM-EDM-CCS-TP were synthesized and cloned into BlueScript plasmid vector by IDT.

1.3. The synthesized genes in step 1.2 were cloned into pET 21b+ expression vector under T7 promoter in the lab.

1.4. The pET-21b vector containing the genes encoding NLS-DCM-EDM and NLS-DCM-EDM-CCS-TP were expressed in BL21 (DE3)pLysS E. coli expression system. The growth, expression and purification conditions/protocols were optimized for each of the expression products. The purity and expression of the NLS-DCM-EDM and NLS-DCM-EDM-CCS-TP were confirmed by SDS-PAGE and western blot analysis.

Owing to the highly basic nature of the vectors and toxicity to E.coli system, the challenge had to be addressed by successfully screening a stable expression host (E. coli, BL21(DE3)pLysS) and optimizing the temperature, induction, extraction and purification conditions. Also, the storage conditions and the desalting techniques were a major

challenge which was resolved successfully. SDS-PAGE and western blots confirmed the expression of the intended products of right size. The purity of the vectors was > 98%.
For more details, please see appendix A.

Task 2- Characterize the vectors using physicochemical and biological assays (months 6-12)

To perform the task:

2.1. The exact molecular weight of the expressed vector was determined by MALDI-TOF. The ability of cathepsin D in cleaving its substrate was examined and optimized. The pH dependent hemolytic activity of the proposed vector was also studied.

2.2. All vectors were complexed with pDNA (pEGFP) to form complexes and characterized in terms of size and charge by Malvern zeta sizer and shape by Transmission Electron Microscope.

2.3. The vector/pDNA complexes were examined in terms of protection of plasmid DNA from serum endonucleases, breast cancer specific cell targeting, transfection efficiency and cell toxicity. Cell transfection studies were performed using breast cancer (ZR-75-1) and normal human mammary cells (MCF-10A). Cells were transfected with the vector/pEGFP (encodes green fluorescent protein) complexes in the presence of chloroquine, bafilomycine and Nocodazole. The transfection efficiency was measured using a flow-cytometer. (months 9-12)

The mass spectroscopy confirmed the size of the vectors which was in agreement with the expected theoretical values. The cathepsin D substrate was accessible for cleavage by the enzyme. The vector was able to lyse cell membranes only at low pH. Protection of pDNA from serum nucleases was observed by gel retardation assay. The mean size of the particles was ≈ 59 nm. The particle size was stable over a 48 h period at room temperature or at 4°C. Transfection studies revealed that the nanoparticles were able to selectively target the ZR-75-1 breast cancer cell line while ignoring the normal breast cancer cell line MCF-10A. Presence of chloroquine had no significant change in transfection efficiency indicating that the fusion peptide EDM was efficient in endosome membrane disruption and release of the trapped nanoparticles. The microtubule mediated localization of nanoparticles into the nucleus was disrupted on addition of Nocodazole proving that the translocation to nucleus was mediated by NLS. Transfection efficiency of 16% was obtained at a Vector to DNA ratio of 10 as detected by flowcytometry.
For more details, please see Appendix A.

Taking advantage of bystander effect, this level of transfection efficiency was sufficient to kill almost 80% of cancer cells by administering one dose of 1 µg piNOS (encodes

nitric oxide synthase) to ZR-75-1 breast cancer cells. A full report on the therapeutic efficacy of the developed vector will be submitted by the end of the contract.

Task 3- Writing manuscripts, final report and dissemination of the data in conferences (months 11-12).

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1 manuscript is submitted for publication. 3 abstracts are published. One more manuscript is in preparation. Please see appendices.

Key Research Accomplishments

- **a)** Developed reproducible expression and purification protocols for the production of highly basic vectors in E.coli system.
- **b)** Engineered a vector composed of five independent functional domains of diverse origin into a complex designer vector while preserving the functionality of each motif under one-domain-one-function concept.
- **c)** Proposing a model for the intracellular trafficking of the vector/pDNA nanocarriers and validation.
- **d)** Full vector characterization and evaluation demonstrating the vector's breast cancer cell specificity and high efficiency with no significant toxicity.

Reportable Outcome

A) Manuscripts: A manuscripts have been prepared and submitted for publication. (Please see appendix A)

B) Presentations: Three abstracts have been published and three posters presented. (Please see appendix B, C and D)

C) Grant application: Based on the obtained results, a grant application is prepared which will be submitted to the Association of International Cancer Research on October 30th, 08. The requested direct cost for this grant application is ~$300,000.

D) Training: A senior postdoctoral fellow with experience in molecular biology and biochemistry was hired. He received training in vector development, vector characterization, mammalian cell culture and transfection, and targeted breast cancer gene therapy.

Conclusions

We have successfully demonstrated that a multi-domain designer vector with complex chimeric architecture can retain individual functionality of its constituents. This vector is fully functional with ability to target breast cancer cells with high specificity, overcome the biological barriers associated with targeted gene transfer, and mediate efficient gene transfer. We have gained a valuable experience in development of complex designer

vectors despite the lack of "rational-design" database for *de novo* protein architecture evolution. This would allow creation of efficient and targeted systems that can be fine tuned for various gene delivery needs. The reported designer biomimetic vector can be modified, equipped with a variety of targeting motifs, and programmed to transfer genes to various breast cancer cell types overexpressing different biomarkers. The ability of the developed vector to efficiently target and kill breast cancer cells *in vitro* is investigated with exciting results. The next logical step is to evaluate the efficacy of the system in animal models which will be contingent on the availability of the funds.

References

None

Appendices

A. Submission of a manuscript for publication.

B. Sriramchandra. S. Mangipudi, Arash Hatefi, **Engineering Biomimetic Vectors with Chimeric Architecture for Targeted Gene Therapy.** 6th International Nanomedicine and Drug Delivery Systems; Toronto, Canada. poster # 29

C. Sriramchandra S. Mangipudi, Arash Hatefi, **Development of a Recombinant Non-Viral Vector for Targeted Breast Cancer Gene Therapy.** Controlled Release Society 35th annual meeting, New York, NY, USA, poster #885

D. Sriramchandra S. Mangipudi, Arash Hatefi, **Development of a Nature-Inspired Vector for Targeted Breast Cancer Gene Therapy.** DOD Era of Hope meeting 2008, Baltimore, MD, USA, Poster # P50-3

Development of a Novel Designer Biomimetic Vector for Targeted Gene Transfer to Breast Cancer Cells

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ABSTRACT

Using component integration approach, several subsystems of diverse biological origin were integrated onto a modular platform in order to carry diverse functions of an efficient gene delivery vehicle. A biomimetic vector was genetically engineered to contain at precise locations: a) an adenovirus μ peptide to condense pDNA into nanosize particles, b) a combinatorially-screened synthetic peptide to target breast cancer cells and enhance internalization of nanoparticles, c) a pH-responsive synthetic fusogenic peptide to disrupt endosome membranes and facilitate escape of the nanoparticles into the cytosol, and d) a nuclear localization signal from human immuno-deficiency virus for microtubule mediated transfer of genetic material to the nucleus. The vector was characterized physicochemically and biologically and the results demonstrated that a fully functional vector can be engineered to target breast cancer cells with high specificity, mimic virus characteristics and overcome the biological barriers associated with targeted gene transfer. This novel approach in vector design would allow creation of efficient targeted vectors that can be fine tuned for various gene delivery needs.

INTRODUCTION

Gene therapy is perceived as a ground-breaking new technology with the promise to cure almost any disease, provided that we understand its genetic basis. However, enthusiasm has rapidly abated as multiple clinical trials failed to show efficacy. The limiting factor seems to be the lack of a suitable delivery system to carry the therapeutic genes safely and efficiently to the target tissue [1]. Gene-transfer technology is still in a nascent stage owing to several inherent limitations in the existing delivery methods. While lipoplexes provide high transfection efficiency, their reproducibility and cytotoxicity remain a major concern [2]. On the other hand, cationic polyplexes are robust and biocompatible but they are marred by poor gene-transfer efficiency [3]. In contrast, viral vectors are the gene-delivery vehicles of choice particularly due to their superior efficiency and the enormous possibility for recombinant engineering. However, safety issues and toxicity have limited their use for systemic gene therapy [4].

What has been long sought after is a technology which combines the biocompatibility of ployplexes, efficiency of lipoplexes and the engineer-ability of viruses in developing effective gene-transfer technology. An alternative biomimetic non-viral approach is at horizon but not without its own package of challenges that must be overcome before making real advances in developing a feasible technology. A significant amount of preliminary groundwork has been done addressing the feasibility concerns of this approach [5-9].

Herein, we report a hybrid vector which is an ensemble of molecules of biological and combinatorially-screened synthetic origins for targeted gene transfer. The first generation of this class of gene delivery systems, namely designer biomimetic vectors (DBVs), is based on a one-domain – one-function architecture concept.

In contrast to viruses, wherein, their complex assembly architecture delegates different functional domains to specific protein subunits dictating the packaging of virus-specific DNA, the non-viral DBV reported here embeds several functional domains derived from as many different natural or synthetic motifs onto a single biomacromolecule for indiscriminate packaging of any passenger DNA. By shifting the order of the functional domains, several versions of the DNA transporter were designed to screen the active candidate resulting in the optimum sequence shown in **Fig. 1a**. Using this approach, the functional propensity of the vector was scripted into the primary sequence structure so as to carry an inherent information-base needed to perform an array of self-guided functions. These include: a) efficient condensation of the plasmid DNA into deliverable nanoparticles by a DNA condensing motif (DCM) obtained from adenovirus μ peptide [10], b) cell-specific delivery of the nanoparticles using a combinatorially screened cyclic targeting peptide (TP), c) endosomal disruption by an engineered pH-responsive endosome disrupting motif (EDM) [11] mimicking influenza virus fusogenic peptide, and finally d) localization of the gene to the nucleus for efficient gene expression by a nuclear localization signal (NLS) obtained from human immuno-deficiency virus type 1 (HIV-1) [12]. Because of the cyclic characteristic of the targeting peptide, the DBV was engineered to stably exist as a monomer and dimer **(Fig 1a)**. A cathepsin D substrate is also engineered in the vector structure to facilitate dissociation of the targeting peptide from the vector inside endosomes where cathepsin D is abundant [13]. The proposed model for the intracellular trafficking of the vector is shown in **Fig. 1b.**

We hypothesize that the proposed DBV is able to condense pDNA into nano-size particles, protect pDNA from degradation by serum endonucleases, target model cancer cells and internalize via receptor-mediated endocytosis, disrupt endosome membranes and escape into the

cell cytoplasm, exploit microtubules to translocate the genetic material into the nucleus, and mediate gene transfer.

RESULTS AND DISCUSSION

During the evolution process, nature has designed various motifs that can efficiently condense DNA (e.g., histones and virus DNA condensing peptides) [10, 14], disrupt endosomes (e.g., adenovirus fusogenic peptide and mellitin) [15, 16], or actively translocate genetic materials to the cell nucleus (e.g., adenovirus nuclear localization signal) [17]. Inspired by nature, the objective of this research was to develop a new strategy to create pseudo-viral assembly architectures (nanoparticles) with selective capabilities that mimic desirable features of viruses. Packaging of multiple motifs with diverse characteristics while preserving their functionality in one vector is undoubtedly the major challenge of this approach. Using genetic engineering techniques, a multi-domain biomimetic vector was designed and synthesized.

The gene encoding the vector, namely DBV, was cloned into a pET21b expression vector to make pET21b:DBV and the sequence of the gene was verified by DNA sequencing. The results showed no signs of deletion or mutation during the cloning process. The expression system was transformed into *E. coli* BL21 (DE3) plysS and DBV was expressed at a 4mg/liter yield. The purity and expression of DBV in both monomer (molecular weight: 11.4 kDa) and dimer (molecular weight: 22.8 kDa) configurations with more than 98% purity were confirmed by SDS-PAGE and westernblot analysis, respectively **(Fig. 2a)**. We have previously established and reported the technology to produce highly cationic vectors in *E.coli* under similar conditions [8, 9].

It was previously mentioned that the cathepsin D substrate in the vector structure is engineered to facilitate dissociation of the targeting motif from the vector inside endosomes. The vector was incubated with the cathepsin D to evaluate the accessibility of the substrate to the enzyme. We used the dimer configuration as the starting point and the results demonstrated that cathepsin D is able to recognize its substrate, digest the dimer from two positions, and give rise to two bands on the SDS-PAGE gel at 11.2 and 8.7 kDa **(Fig. 2b)**. This was expected as the dimer contains two cathepsin D substrates in its structure. The results show that the probability for the vector to shed the targeting motif inside endosomes exists. Because the targeting motif is designed in the vector structure to facilitate internalization of nanoparticles, its removal after receptor mediated endocytosis could result in better exposure of the EDM on the nanoparticle surface and facilitate interaction with the endosomal membrane **(Fig. 1c, steps 1 to 4)**.

We further characterized the vector in terms of its pH and concentration dependent hemolytic activity by incubating the vector with red blood cells. It was observed that the hemolytic activity of the vector significantly increased as pH decreased **(Fig. 2c)**. The endosome disrupting motif used in this study was first developed by Monsigny's group [11], namely 5HWYG. It mimics the endosome disrupting activity of fusogenic peptide in influenza virus but at a pH closer to 6.9. This would allow the EDM to change conformation into α-helical structure, fuse with the early endosome membranes, and escape into the cytoplasm. This feature of EDM, the pH responsiveness, is extremely important as it minimizes the possibility of causing cell damage while circulating in the blood stream. We also observed that the hemolytic activity of the EDM was concentration dependent **(Fig. 2c).** Therefore it can be construed that as the number of vector molecules used to complex with one pDNA molecule increases, the endosome disrupting efficiency of the nanoparticles could increase. So far, we have characterized the vector in terms

of expression, purity, hemolytic activity, and digestion by cathepsin D. The next logical step is to examine the ability of the vector to complex with pDNA followed by physicochemical and biological characterization.

In the vector structure we have utilized arginine rich adenovirus μ peptide [18] to complex with pDNA and form condensed nanosize particles. To examine the extent of pDNA condensation, vectors (dimer and monomer) were complexed with pEGFP and characterized in terms of size by dynamic light scattering technique and shape by transmission electron microscopy. The TEM images show that the vector/pEGFP complexes are compact spherical nanoparticles **(only dimer is shown; Fig. 3a).** The results of particle size analysis showed that both dimer and monomer were able to efficiently condense pEGFP into nanoparticles with average sizes below 100nm at N:P ratios between 4 to 10 **(Fig. 3b)**. It has been shown that nanoparticles in this size range are suitable for receptor mediated endocytosis [19].

Nanoparticles formed at various N:P ratios were further characterized in terms of their ability to mediate gene transfer into ZR-75-1 model breast cancer cell line. This cell line was selected because the model targeting peptide used in the vector structure is developed through combinatorial screening to specifically target this cell line with minimal binding affinity towards normal mammary cells [20]. As a negative control MCF-10A normal mammary cell line was used to demonstrate cell specific targeted gene transfer. ZR-75-1 cells were transfected with the vector/pEGFP complexes formed with monomer and dimer at various N:P ratios. While GFP expression was observed for both monomer and dimer at N:P ratios of 8, 9, 10, and 11 (data not shown), the highest levels of transfection efficiency were observed at N:P 10 **(Fig. 4a)**. In this study, the word “transfection efficiency” encompasses both percent transfected cells and overall total green fluorescent protein expression. As a positive control we used lipofectamine to

validate the transfection protocol. Commercially available transfection reagents such as lipofectamine (non-targeted) are usually formulated to flocculate into large size particles so that they can precipitate readily onto the cells surface for maximum transfection efficiency. Such non-targeted systems may not be suitable for systemic gene delivery due to their non-specific binding to normal cells. While the percent transfected cells by dimer and lipofectamine was not statistically different, the level of green fluorescent protein expression for dimer was significantly higher than lipofectamine. We did not observe any significant difference between the transfection efficiency of monomer versus dimer. As a result, we proceeded with the characterization of dimer only which holds a more complex architecture.

Nanoparticles formed with the dimer at N:P 10 were also characterized in terms of stability and protection of pDNA in the presence of serum. The results showed that these particles were stable in the absence and presence of 10% serum **(Fig. 4b, lanes 2 and 3)** for an extended period of time and effectively protected pDNA from degradation by the serum nucleases **(Fig. 4b, lane 4)**.

We also performed an inhibition assay to demonstrate internalization of nanoparticles via receptor-mediated endocytosis. This was evaluated by pre-treatment of ZR-75-1 cells with the targeting peptide to saturate the receptors followed by transfection of the cells with vector/pEGFP complexes. The results of this assay revealed that as the concentration of the targeting peptide (competitive inhibitor) increased, the levels of gene expression decreased **(Fig. 5)**. At 2.8 nM ligand concentration, almost complete suppression of the transfection was achieved. The inverse relationship between ligand concentration and total GFP expression indicates that addition of the targeting peptide to the media could block internalization of targeted nanoparticles resulting in lower levels of gene expression.

To demonstrate cell-specific delivery of the nanoparticles, the vector/pEGFP complexes at optimum N:P ratio of 10 was also used to transfect MCF-10A normal mammary cells. The results showed that the levels of gene expression were undetectable **(Fig. 6a)**. This could be due to the absence of receptors on the surface of MCF-10A cells for the targeting peptide. This suggests that the targeting peptide facilitated receptor-mediated internalization of the nanoparticles in ZR-75-1 cells with no significant internalization in normal mammary cells. This finding was expected as the targeting peptide in the vector structure was developed by the bacterial display system (combinatorial screening) to specifically target ZR-75-1 but not MCF-10A cells [20].

We then asked the question whether the presence of EDM in the vector structure could play a role and have a significant effect on the endosomal escape and transgene expression. The designed fusogenic peptide is expected to effectively increase the delivery of pDNA into the cytosol via membrane destabilization of acidic endocytotic vesicles containing vector/pDNA complexes. This was assessed by transfecting ZR-75-1 cells in the absence and presence of bafilomycin A1 and chloroquine. Choloroquine is a buffering agent known to disrupt the endosomal membrane by increasing the pH of the endosome environment [21]. In contrast, bafilomycin A is an inhibitor of vacuolar ATPase endosomal proton pump which prevents the escape of the cargo into cytosol by inhibiting the acidification of the endosome environment [22]. When ZR-75-1 cells were transfected in the presence of bafilomycin, the transfection efficiency was significantly reduced from 45,061 ± 7,828 to 4,064 ± 2,682 light units highlighting the fact that the acidification of the endosomal compartment is necessary for the escape of the nanoparticles into cytosol **(Figs 6a and b)**. These results in combination with the results obtained from the hemolysis assay suggest that EDM played a significant role in

enhancing gene expression due to its pH-dependent fusogenic activity. Interestingly, in the presence of chloroquine, no significant increase in transfection efficiency was observed **(Figs 6a and b)**. This observation supports the hypothesis that the EDM motif could preserve its functional integrity and assist the nanoparticles to efficiently escape from the endosomes into cytosol. It is plausible that this significant endosome disrupting activity is the result of additive or synergistic effects of fusogenic peptide and histidine residues in the vector structure. Because we have designed a histag in the vector sequence to facilitate purification of the vector from the *E.coli*, it could have contributed to the disruption of endosomes via the proton sponge effect [23, 24]. It is also noteworthy that EDM and DCM contain 5 and 3 histidine residues in their sequences, respectively. Therefore, the presence of 14 histidines out of 95 amino acid residues in the vector sequence along with the fusogenic peptide could have been the reason for efficient endosome membrane disruption. More in depth studies on this finding are needed to better understand this phenomenon.

Although there is limited understanding of the cellular and molecular mechanisms involved with synthetic vector mediated gene transfer, transfection efficiency appears to be essentially limited by inefficient trafficking of DNA to the site of gene transcription in the nucleus [25]. To overcome this obstacle, we utilized the NLS in the *Rev* protein (residues 35-51) of Human Immunodeficiency Virus (HIV) and engineered the signal sequence at the vector's N-terminal to promote the transport and accumulation of nanoparticles inside the nucleus. ZR-75-1 cells were transfected in the presence and absence of nocodazole, a reagent known to depolymerize microtubule structures, to examine the effect of NLS on the translocation of pDNA to the nucleus via microtubules [26]. The results revealed significant reduction in gene expression from 45,061 ± 7,828 to 5,191 ± 2,334 light units when microtubule networks were

disrupted **(Figs 6a and b)**. Therefore, it can be deduced that the nanoparticles exploited microtubules to reach the nucleus. Consequently, this higher level of gene expression can be attributed to the active presence of NLS in the vector structure.

So far, we have examined each motif in the vector structure and have shown that by correct positioning in the vector backbone, the functionality of each motif can be preserved. This can be explained by the fact that during the pDNA condensation process, hundreds of vector molecules participate to condense one molecule of pDNA. For example, at an N:P ratio of 10, approximately 2250 vector molecules each containing 42 positively charged residues are employed to condense one pEGFP molecule (9462 negative charges). Therefore, it is probable to have a fraction of fully functional targeting motifs, NLSs, and EDMs in the vector/pDNA complex architecture. Thus, the nanocomplex is rendered ready to bind to the receptors, fuse with the endosome membranes, and utilize the microtubules for active translocation of genetic material to the cell nucleus.

Besides high levels of gene expression, vector related toxicity is also a major concern. To examine vector toxicity, ZR-75-1 cells were incubated with the vector alone or vector in complex with pEGFP. The results exhibited no significant cell toxicity in either case at a concentration of up to 150 µg/ ml **(Fig. 6c)**.

One last question that we asked was why all cells were not transfected, even though the vector seems to have overcome all the known cellular barriers. Beside the administered pDNA dose, the answer could be as simple as the fact that the abundance of entry gates (i.e., receptors) on the surface of cancer cells dictates the number of particles that can be internalized. Viruses such as adenovirus are a good example as they are able to transfect various cell lines with different transfection efficiencies depending on the number of coxsackie adenovirus receptor

present on the surface of the cells [27]. In addition, not all ZR-75-1 cancer cells over-express required receptors for the internalization of the nanoparticles because cancer cell populations are usually heterogeneous. Nonetheless, other more complex reasons could be involved which invites for more mechanistic studies to unravel the mysteries of efficient gene transfer.

CONCLUSION

We have demonstrated that a multi-domain designer vector with a complex chimeric architecture can retain individual functionality of its constituents. There is an increasing body of evidence that proteins are more flexible than previously thought and can retain functional integrity to multiple conformations [28]; thus, boosting the possibility of successful *in vitro* evolution of complex designer macromolecules. This would allow creation of efficient and targeted systems that can be fine tuned for various gene delivery needs. The reported DBV is engineered to readily swap the targeting peptide or add one or more accessory helper motifs. Such systems can be equipped with a variety of targeting motifs and used to transfer genes to various cell types with applications in gene therapy of cancer, cardiovascular disease, wound healing, and many other diseases.

MATERIALS AND METHODS

Cloning, expression, and purification of DBV

The gene encoding DBV was designed and then custom synthesized at Integrated DNA Technologies, Inc. and placed in pZErO plasmid. The resident synthetic DBV gene flanked on either side by NdeI and XhoI restriction sites was cloned into a pET21b expression vector under

the control of a T7 promoter. The resultant expression construct pET21b:DBV was tested for its fidelity to the original design by DNA sequencing.

The pET21b:DBV expression vector was transformed into *E. coli* BL21(DE3) pLysS. Starter cultures, 5 ml, were then grown in LB media at 37°C overnight with 50 μg/ml carbenicillin. Circlegrow media (MB Biomedicals, Solon, OH), 500 ml, was inoculated with the starter culture and allowed to grow at 30°C until the absorbance reached 3.0 at 600nm. The culture was inducted with 0.4 mM IPTG for 2 hours. The culture broth was centrifuged at 6000 rpm for 7 min at 4°C and the pellet frozen until further use.

The pellet was suspended in lysis buffer containing 100 mM NaH_2PO_4, 10 mM Tris-HCl (pH 8.0), and 6M guadinium hydrochloride and the lysate was centrifuged at 18000 rpm for 60 min. For purifying the dimer no reducing agent was used, whereas for the purification of the monomer 12 mM 2-mercaptoethanol was used in all buffers. The cleared lysate supernatant was adjusted to 12 mM immidazole and 0.5 ml of Ni-NTA resin equilibrated in the same buffer was added. The expressed DBV was allowed to bind in a batch mode for 60 min at room temperature. The tubes were centrifuged at 1000g for 5 min and the supernatant discarded. The resin was loaded in a 0.8 x 4 ml BioRad PolyPrep chromatography column, washed with 25 ml of lysis buffer supplemented with 15 mM immidazole, and eluted with 250 mM immidazole. The DBV rich elution was stored in 50% glycerol at -20°C. Before use, the buffers were exchanged and replaced with bis-tris propane buffer (10mM, pH 7) and 5mM NaCl. The purity and expression of the DBV were determined by SDS-PAGE and western blot analysis (monoclonal anti-6XHis), respectively.

Recognition of cathepsin D substrate by cathepsin D protease

Cathepsin D enzyme from human liver was purchased from Calbiochem (Gibbstown, NJ) and dissolved in 0.1 M Glycine-HCl, 0.5% Triton X-100, and NaCl to bring the final ionic strength to 150 mM. One unit of enzyme was added to 10μg of vector in a volume of 50 μl and incubated at 37°C for 3 h. Samples were boiled at 95°C for 5 min and loaded onto 12% SDS-PAGE gel.

Hemolysis assay

Two milliliters of sheep red blood cells (Innovative Research, Novi, MI) was washed several times with phosphate buffered saline (PBS). Cell numbers were adjusted to 3.2 x 10^8 cells ml^{-1} in either PBS at pH 7.0 or 50 mM acetate buffer at pH 5.0, adjusted to physiological ionic strength with NaCl. The cell suspension in either buffer was supplemented with 1, 10, 20 or 30 μg of the vector. The mixture was incubated at 37°C for 1h, centrifuged and the absorbance of the supernatant was measured at 541 nm. Triton X-100 (1%) was used as the positive and phosphate and acetate buffers as negative controls. The percentage of hemolysis for the test groups is reported as relative to fully lysed cells by Triton X-100 (defined as 100%). Data is reported as mean ± s.d., n=3. The statistical significance was tested using t-tests ($p<0.05$).

Particle size analysis

Vectors were complexed with plasmid DNA encoding green fluorescent protein (pEGFP Clontech, CA, USA) at various N:P ratios (the molar ratio of positively charged nitrogen atoms to negatively charged phosphates in pDNA). Complexation was achieved by adding various amounts of vector solution in bis-tris propane buffer (pH 7) to an equal volume of buffer containing 1 μg pEGFP. The mean hydrodynamic particle size measurements for vector/pDNA

complexes were performed using Dynamic Light Scattering (DLS) by a Malvern Nano ZS90 instrument and DTS software (Malvern Instruments, UK). The data is reported as mean ± SEM, n=3.

Particle stability in serum

The stability of the nanoparticles in the presence of serum was examined using gel retardation assay. In one set of tubes, 1µg pEGFP was complexed with the vector, incubated at room temperature for 15 minutes, and the mobility of pDNA was visualized by ethidium bromide staining and agarose gel electrophoresis. In the second set, particles were formed by complexing 1µg pEGFP with the vector followed by addition of fetal bovine serum (Invitrogen, CA, USA) at the final concentration of 10% (v/v). The complexes were incubated for 30 minutes at 37 ^{o}C in the presence of serum and then electrophoresed on a 1% agarose gel. The pDNA mobility was visualized by ethidium bromide staining.

To evaluate the ability of the vector in protecting pDNA from endonucleases, complexes were formed at an optimal N:P 10 in a microfuge tube and incubated with serum for 90 minutes. Subsequently, sodium dodecyl sulfate was added (10%) to the tubes followed by heating to 95°C for 5 minutes to release the pDNA from the vector. Samples were electrophoresed on agarose gel and visualized by ethidium bromide staining.

Transmission electron microscopy

The vector/pDNA nanoparticles were prepared at N:P 10 on Formvar coated grids and stained with uranyl acetate for 5 minutes. The nanoparticles were imaged using JEOL 1200 EX II (120 kV) at the University of Idaho Center for Electron Microscopy and Microanalysis.

Cell culture and transfection

ZR-75-1 (ATCC, Manassas, VA) breast cancer cells were seeded in 96 well tissue culture plates in RPMI 1640 supplemented with 10% serum at a density of 5×10^4 cells per well. MCF-10A (ATCC, Manassas, VA) normal human mammary cells were seeded in 96 well tissue culture plates in MEBM complete media at 3×10^3 cells per well. Cells were approximately 80% confluent at the time of transfection. Two hours before transfection, ZR-75 cells were conditioned in serum free media (RPMI-1640 supplemented with Insulin-Transferin-Selinium (ITS), Dexamethasone, and fibronectin human plasma). A 100 µl aliquot of vector/pEGFP complex at various N:P ratios (1 to 16) containing 1 µg pEGFP was added to each well and the plates incubated for 2 h in CO_2 incubator at 37 °C. After 2 h, the media was removed and replaced with RPMI 1640 supplemented with 10% (v/v) serum. When used, chloroquine (100µM), bafilomycin (10nM), or Nocodazole (10µM) were added. The expression of GFP was visualized after 48 hrs by an epifluorescent microscope (Zeiss Axio Observer Z1). The percent transfected cells as well as total green fluorescent protein expression were determined by flowcytometry (FacsCalibur, Becton Dickinson, San Jose, CA). Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used as a positive control to validate the transfection process. Lipofectamine was complexed with pEGFP in Opti-MEM (Invitrogen) as per manufacturer's protocol and used to transfect cells. The data are presented as mean ± s.d., n=3. The statistical significance was evaluated using t-test ($p<0.05$).

Inhibition assay

Cells were seeded at a density of 5 x 10^4 cells per well in a 96 well plate. Aliquots of targeting peptide in serum free media were added at concentrations of 0, 0.07, 0.7, 1.4, 2.8, and 5.5 nM to the wells. The plates were placed at 37°C in CO_2 incubator for 1h with intermittent mixing at intervals of 15 min. Aliquots of dimer (100 µl) in complex with 1 µg pEGFP at N:P 10 were added to each of the wells. The control well with 0 µg/ml concentration received PBS. The measured percent transfected cells for the test groups (pre-treated with targeting peptide) are expressed as percent of the control defined as 100%. The data are shown as mean ± s.d., n=3. The statistical significance was examined using t-test ($p<0.05$).

Cell toxicity assays

This assay was performed in serum free media on ZR-75-1 cells for the vector and vector/pEGFP complexes. ZR-75-1 cells were seeded at a density of 5 x 10^4 cells per well in 96 well plates. Cells were treated with serial dilutions of vector alone or vector in complex with pEGFP for two hours. Subsequently, the media was removed and replaced with fresh RPMI supplemented with 10% serum followed by overnight incubation at 37°C in a humidified CO_2 atmosphere. The control well with 0 µg/ml concentration received PBS. After 48 hours, WST-1 reagent (Roche Applied Science, Indianapolis, IN) was added, incubated for 4 hours, and absorbance was measured at 440 nm. The measured absorbance for test groups is expressed as percent of the control where the control is defined as %100 viable. The data are reported as mean ± s.d., n=3. The statistical significance was determined using a t-test ($p<0.05$).

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1: Schematics of the designer biomimetic vectors and the proposed model for intracellular trafficking mechanisms. **a)** The vector (monomer) is composed of a nuclear localization signal (NLS), a DNA condensing motif (DCM), an endosome disrupting motif (EDM), a cathepsin D substrate (CS), and a cyclic targeting peptide (TP). The dimer is created through the linkage of cystein residues in targeting peptide by disulfide bonds. **b)** Step 1: The positively charged DCM interacts with pDNA and forms condensed nano-size particles. Step 2: The targeting motif binds to the receptors on the surface of ZR-75 cancer cells allowing the internalization of the complexes via receptor mediated endocytosis. Step 3: Targeting motif (TM) separates from the complexes inside endosomes with the help of endogenous cathepsin D enzyme. Step 4: The fusogenic peptide changes conformation at acidic pH into α-helical structure, fuses with the endosome membrane, and facilitates escape of the vector/pDNA complex into the cytosol. Step 5: The NLS motif exploits the microtubules and shuttles the pDNA towards the nucleus. Step 6: If the size of the vector/pDNA complexes is more than 30nm, the complex may end up in the nucleus at the mitosis (M) phase of the cell cycle where the nuclear membrane dissolves. Step 7: If small enough (<30nm), the complex may pass through the nuclear pore complex (NPC). Step 8: Inside the nucleus, the pDNA will be released for transcription.

Figure 2: Expression and characterization of the DBV. **a)** SDS-PAGE (left panel) and western blot analysis (right panel) of purified vector with purity higher than 98%. PM stands for protein marker, M is monomer, and D is dimer. **b)** Digestion of the vector by cathepsin D. PM is protein

marker; lane 1 is undigested vector; and lane 2 is the digested vector. **c)** hemolytic activity of the vector at different pH conditions and concentrations.

Figure 3: Nanoparticle shape and size analysis. **a)** The TEM picture of the spherical nanoparticles formed at N:P 10. **b)** The particle size analysis of vector/pEGFP complexes at various N:P ratios by dynamic light scattering.

Figure 4: Nanoparticle characterization in terms of mediating gene transfer and stability. **a)** Gene transfection efficiency of monomer (N:P 10), dimer (N:P 10), and lipofectamine. pEGFP was used as a model reporter gene. The corresponding nanoparticle size is also reported. **b)** Gel retardation assay of pDNA and vector/pDNA complexes at N:P 10. Lane 1: pDNA in the absence of serum. Lane 2: vector/pDNA complexes in the absence of serum. Lane 3: vector/pDNA complexes incubated with serum. Lane 4: released pDNA from the vector/pDNA complexes after incubation with serum.

Figure 5: Inhibition assay was performed to demonstrate the transfection of cells via receptor-mediated endocytosis. Targeting peptide at various concentrations was used as a competitive inhibitor.

Figure 6: Evaluation of the transfection efficiency and toxicity of vector/pDNA complexes. **a)** Qualitative representation of the MCF-10A and ZR-75-1 cells transfected with vector/pEGFP complexes at N:P ratio of 10 as well as percent transfected cells in the presence of chloroquine, bafilomycin A, and nocodazole. **b)** Quantitative measurements of the total gene expression for

the ZR-75-1 cells transfected with vector/pEGFP complexes at N:P 10 and in the presence of chloroquine (CQ), bafilomycin A, and nocodazole. **c)** WST-1 cell toxicity assay for ZR-75 cells treated with various concentrations of vector alone (rectangle ■) and vector/pEGFP complexes (triangle ▲).

a)

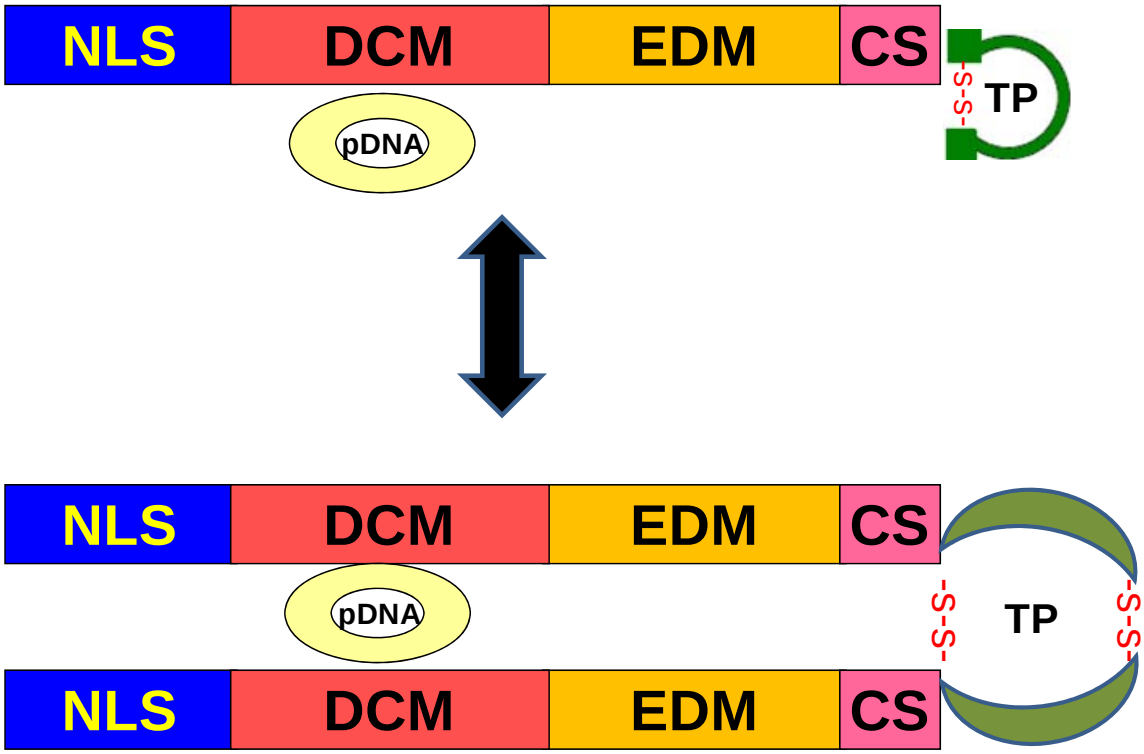
NLS
DCM
EDM
CS
TP
-S-S-
pDNA
NLS
DCM
EDM
CS
pDNA
TP
-S-S-
-S-S-
NLS
DCM
EDM
CS

Figure 1

b)

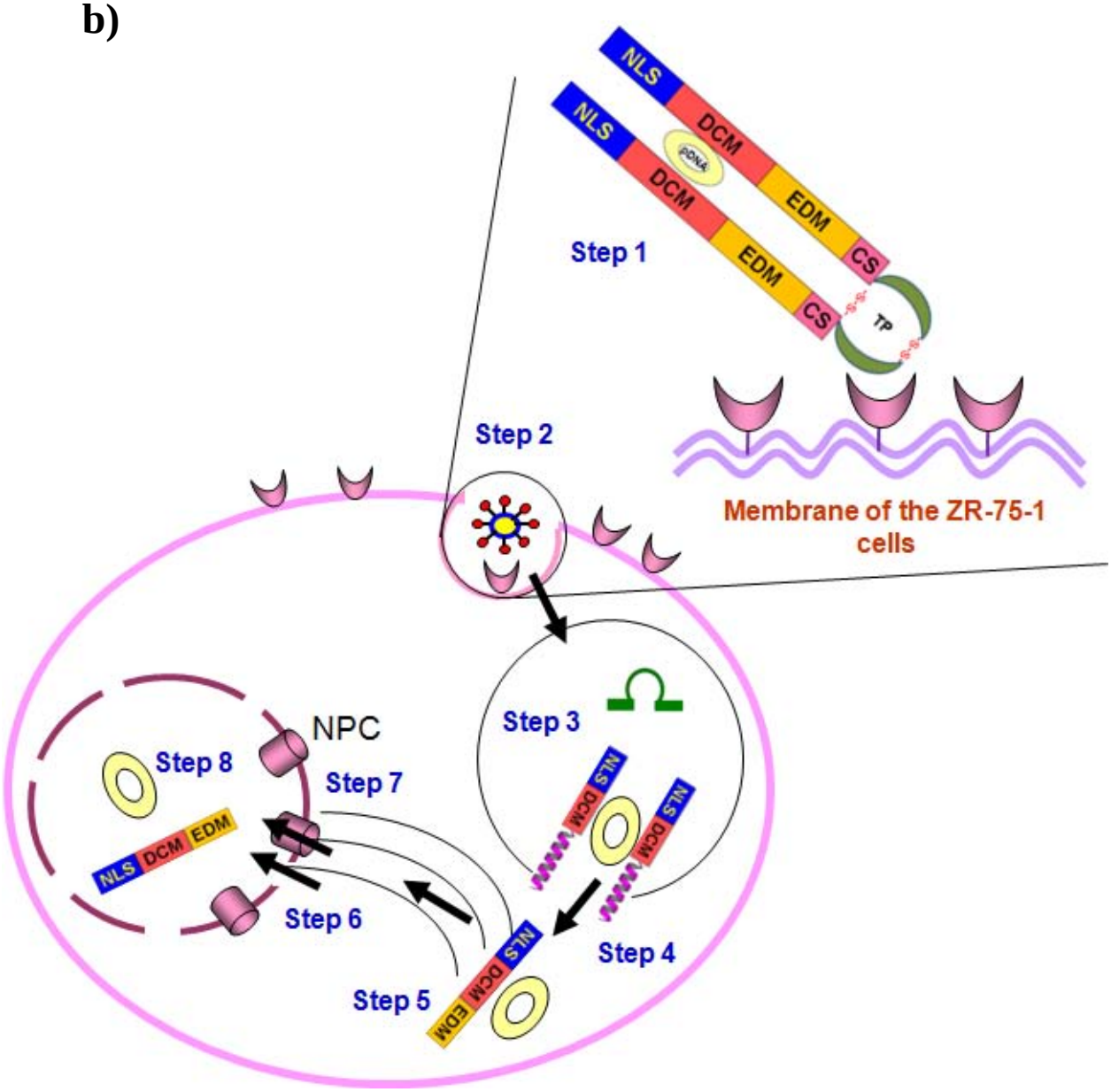
NLS
DCM
EDM
CS
TP
pDNA
Step 1
Step 2
Membrane of the ZR-75-1 cells
Step 3
Step 4
Step 5
Step 6
Step 7
Step 8
NPC

Figure 1b

a)

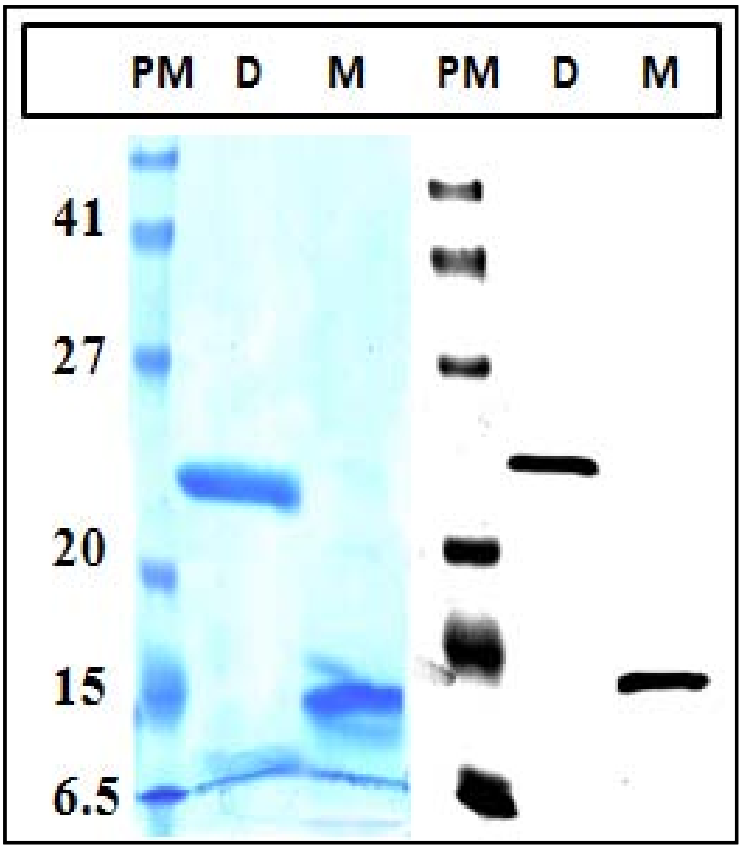

Figure 2a

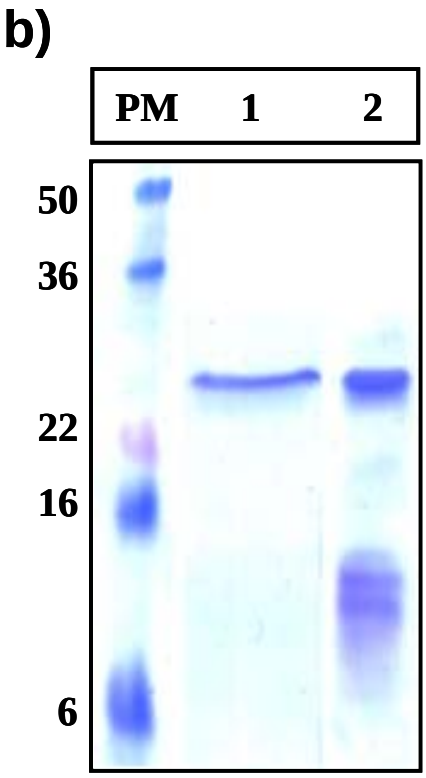

Figure 2b

c)

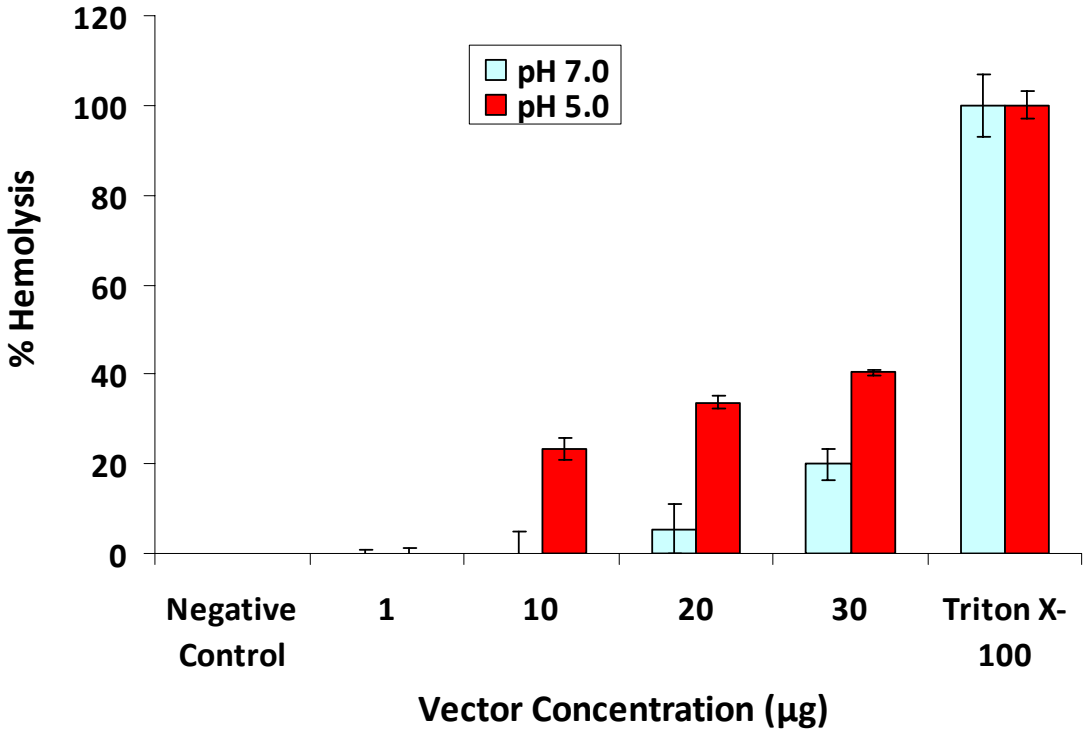
120
100
80
60
40
20
0
% Hemolysis
pH 7.0
pH 5.0
Negative Control
1
10
20
30
Triton X-100
Vector Concentration (µg)

Figure 2c

a)

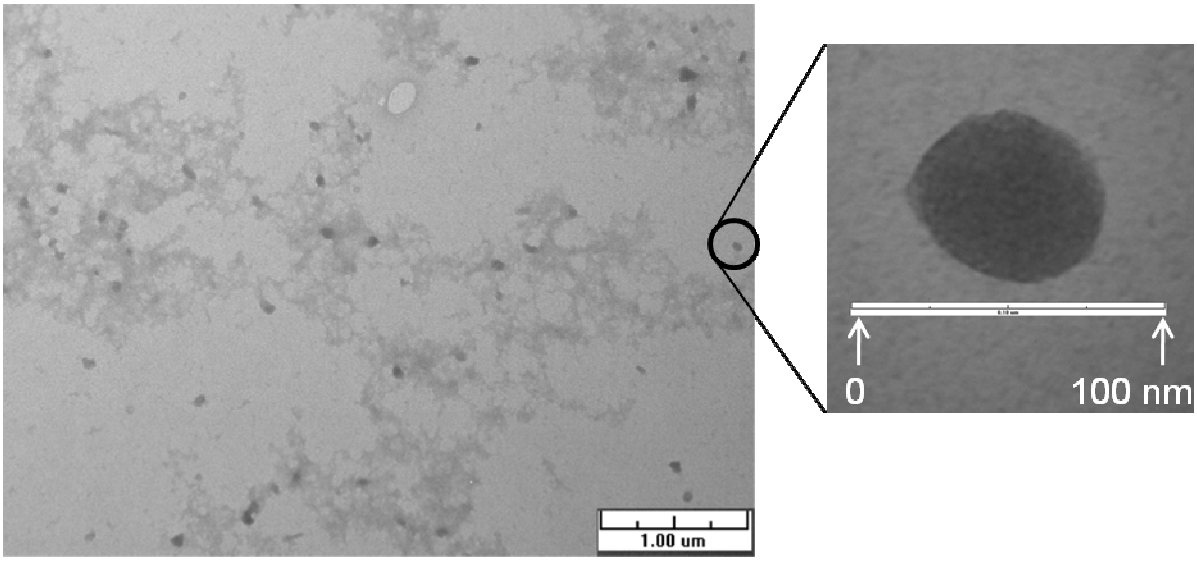

Figure 3a

b)

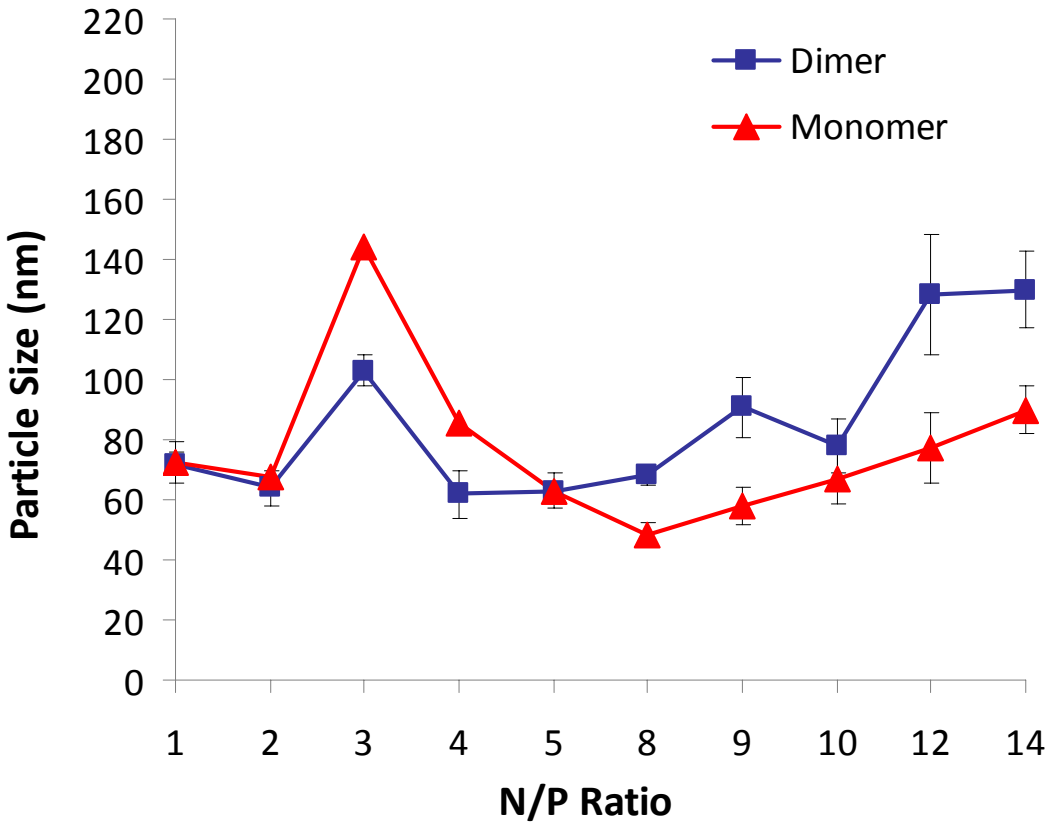
Dimer
Monomer
Particle Size (nm)
220
200
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5
8
9
10
12
14
N/P Ratio

Figure 3b

a)

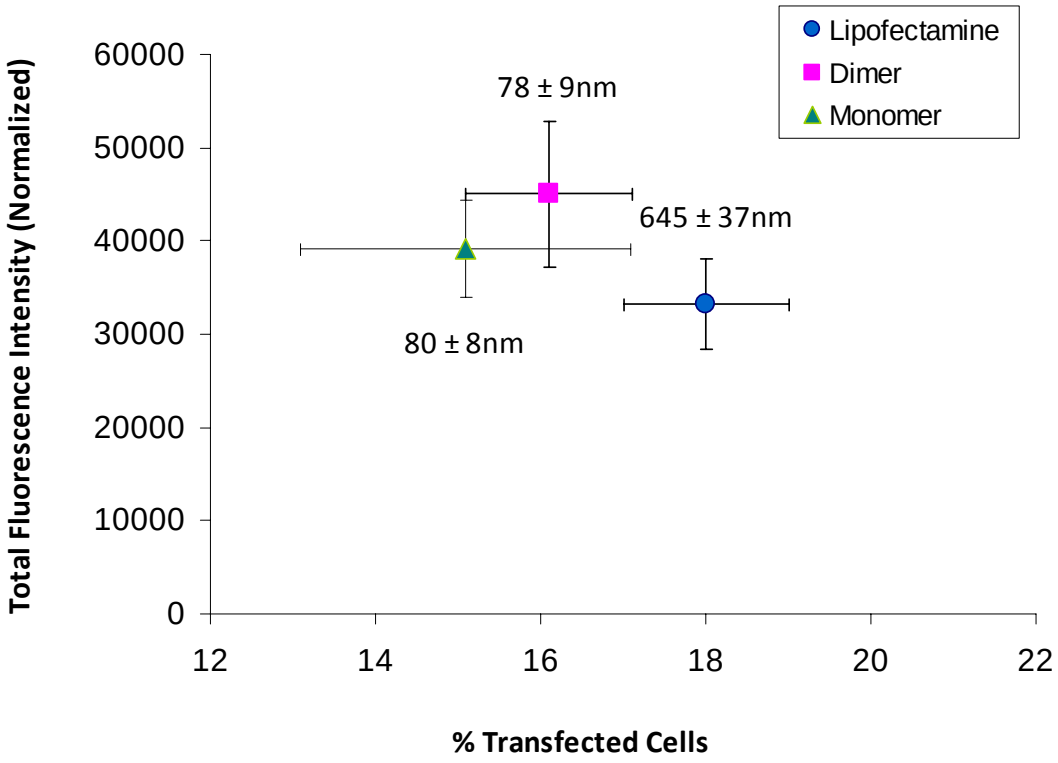
Lipofectamine
Dimer
Monomer
78 ± 9nm
645 ± 37nm
80 ± 8nm
Total Fluorescence Intensity (Normalized)
60000
50000
40000
30000
20000
10000
0
12
14
16
18
20
22
% Transfected Cells

Figure 4a

b)

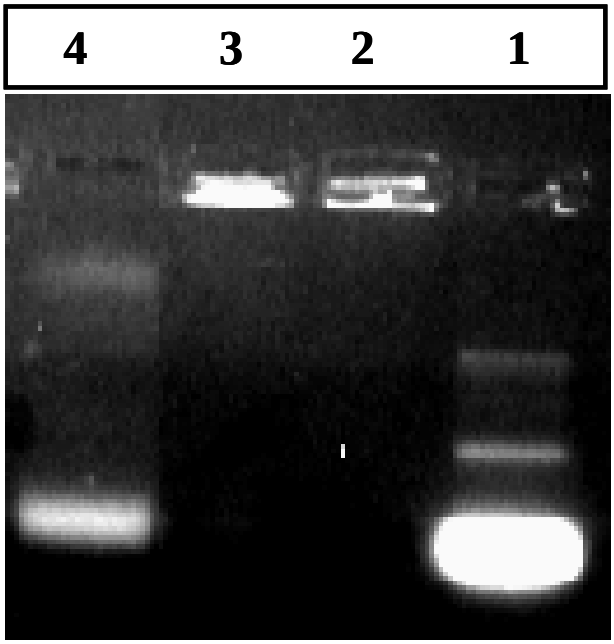

Figure 4b

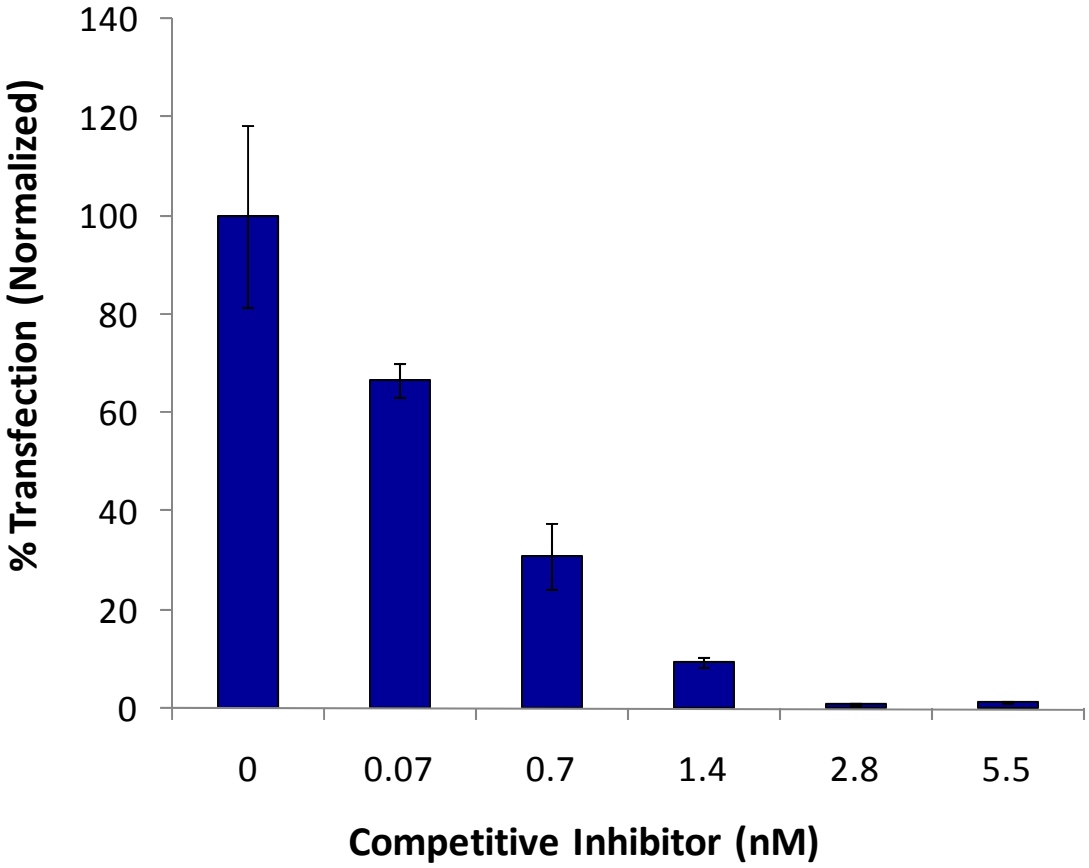
140
120
100
80
60
40
20
0
% Transfection (Normalized)
0
0.07
0.7
1.4
2.8
5.5
Competitive Inhibitor (nM)

Figure 5

a)

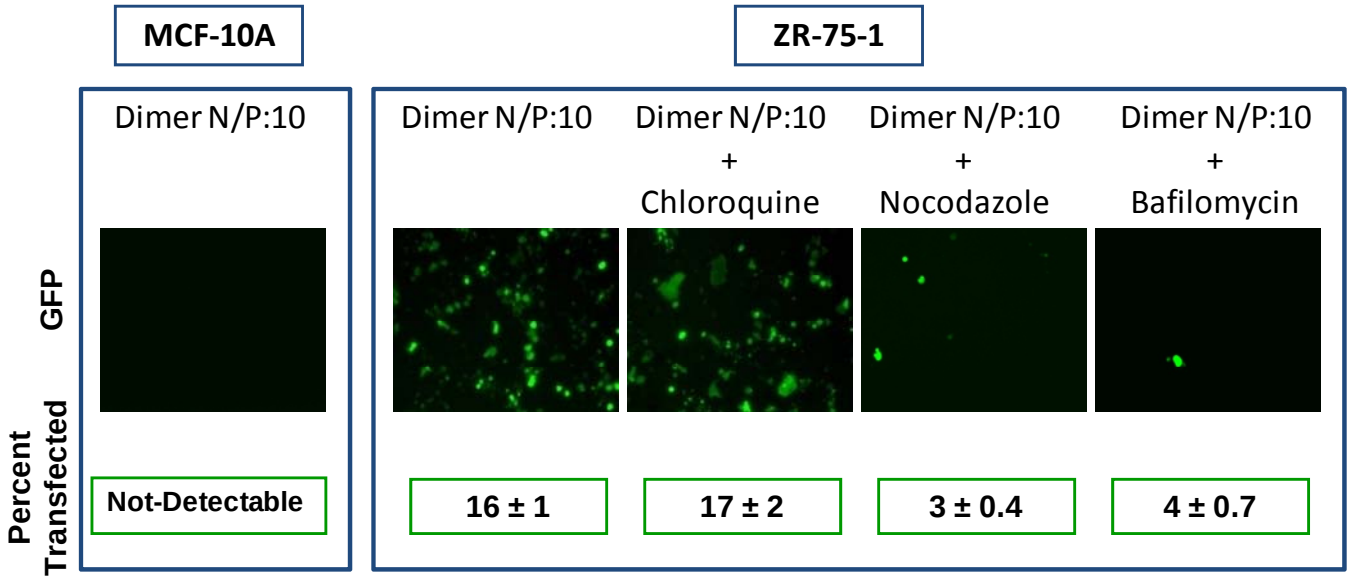
MCF-10A
ZR-75-1
Dimer N/P:10
Dimer N/P:10
Dimer N/P:10
+
Chloroquine
Dimer N/P:10
+
Nocodazole
Dimer N/P:10
+
Bafilomycin
GFP
Percent Transfected
Not-Detectable
16 ± 1
17 ± 2
3 ± 0.4
4 ± 0.7

Figure 6a

b)

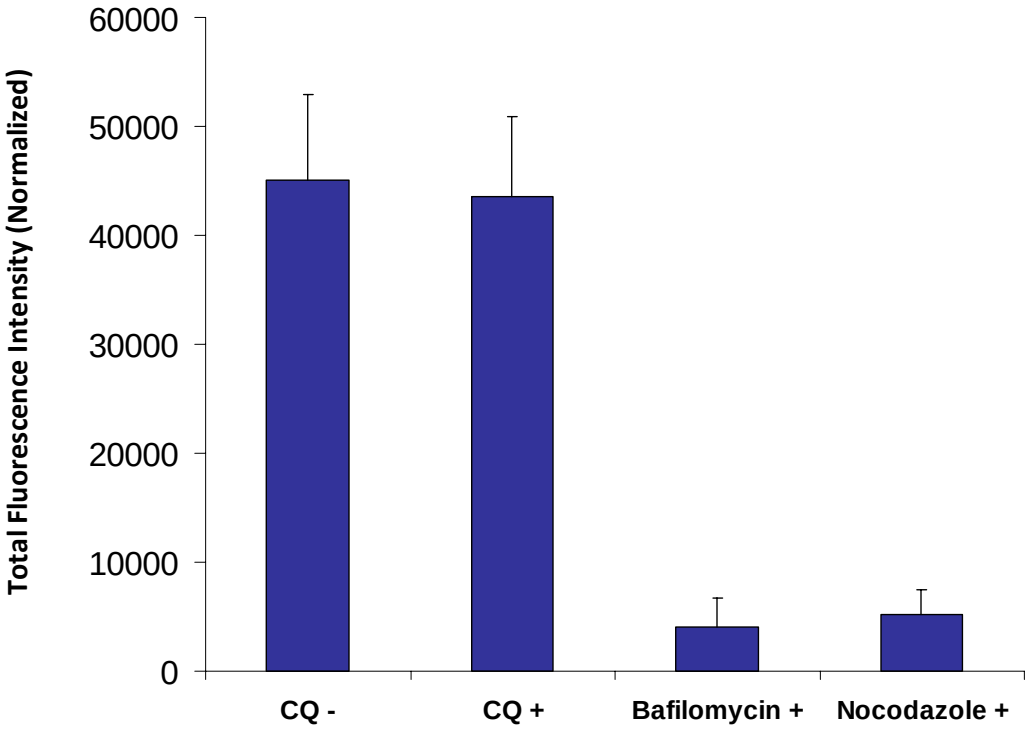
Total Fluorescence Intensity (Normalized)
60000
50000
40000
30000
20000
10000
0
CQ -
CQ +
Bafilomycin +
Nocodazole +

Figure 6b

c)

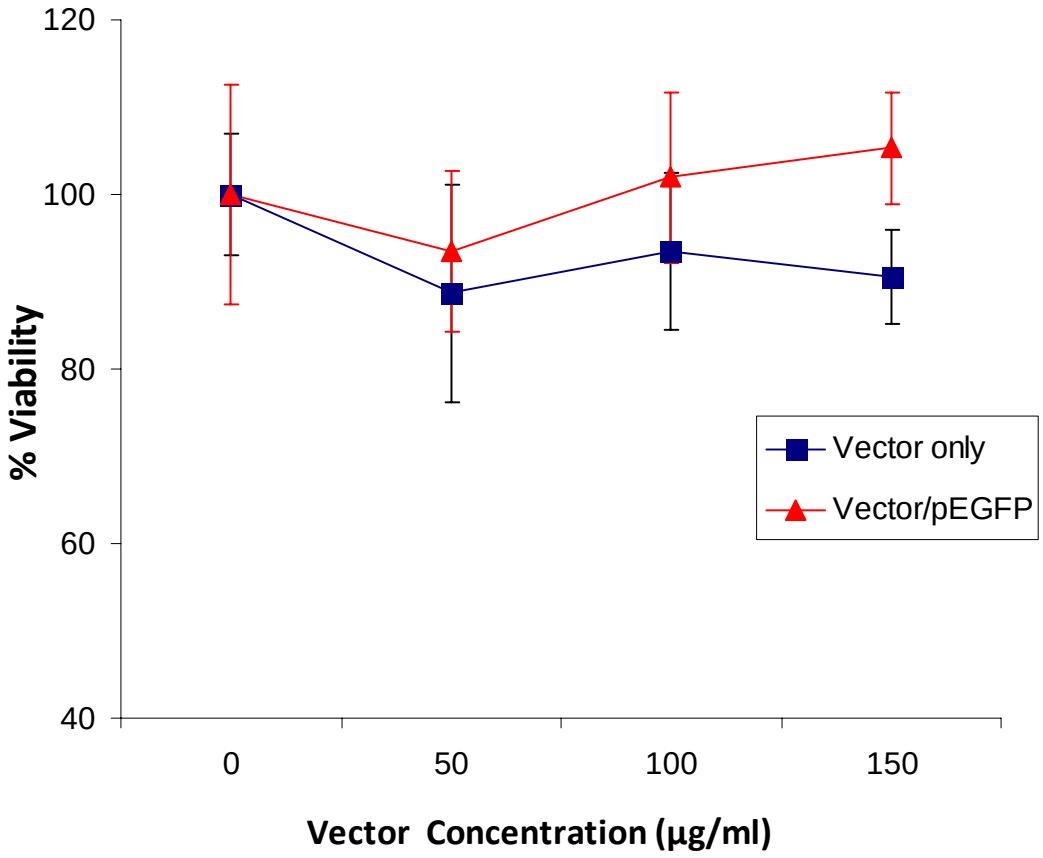
120
100
80
60
40
% Viability
0
50
100
150
Vector Concentration (µg/ml)
Vector only
Vector/pEGFP

Figure 6c

DOD Era of Hope meeting 2008, Baltimore, MD, USA, Abstract # P50-3

Development of a Nature-Inspired Vector for Targeted Breast Cancer Gene Therapy

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Background: The target sites for nucleic acid molecules used in gene therapy are mostly in the cell nucleus. Therefore, for vectors to be maximally effective, they should condense pDNA in order to protect them from serum endonucleases, be recognized and internalized by target cells, promote escape from the endosomal compartment, and direct the nucleic acids towards the nucleus for transcription. A vector was engineered equipped with a DNA condensing motif obtained from adenovirus μ peptide to condense pDNA; an endosome disrupting motif to promote escape of pDNA into cytosol; a nuclear localization signal to facilitate localization of pDNA in nucleoplasm; and a targeting peptide to specifically target human breast cancer cells.

Objective: The objective of this research is to develop a gene delivery system that can target breast cancer cells *specifically* and transfect them *efficiently*.

Methods: Biosynthesis *of the targeted vector:* The nucleotide sequence encoding the vector was synthesized and cloned into a pET21b expression vector. The expression vector was transformed into competent *E. coli* BL21DE3 pLysS cells and expressed. The expressed vector was purified using Ni-NTA column chromatography. The purity and expression was identified by SDS-PAGE and westernblot analysis.
Particle size analysis: Various amounts of vector were complexed with 2μg pEGFP to form nanoparticles and characterized in terms of particle size using Malvern Particle/Zeta sizer.
Cell transfection: ZR-75-1 breast cancer and MCF-10A normal human mammary cells were seeded in 12 well tissue culture plates at 70,000 cells per well. Cells were approximately 80% confluent at the time of transfection. pEGFP was mixed with various amounts of vector for complex formation. The complexes were added to the growth media supplemented with 10% serum. The expression of GFP and percent transfected cells was determined by an epifluorescent microscope.

Results:*Vector biosynthesis:* The westernblot analysis using anti-histag antibody confirmed the expression of the vector. The SDS-PAGE results demonstrated that the purity of the vector was >98%.

Particle size analysis: The results of the vector/pDNA complexation studies showed that the purified vector was able to condense pDNA into nanosize particles with 78± 8 nm size suitable for cellular uptake.

Transfection studies: The results demonstrated that the vector/pEGFP complexes were able to transfect 45 ± 8 % of the ZR-75-1 cells in the presence of serum. Approximately 5% of the MCF-10A cells were transfected which could be due to the non-specific uptake of the particles.

Conclusion: It was concluded that the proposed vector is able to condense pDNA efficiently into nanosize particles and transfect breast cancer cells efficiently with minimal non-specific uptake by normal mammary cells. Additional optimization studies are currently under investigation.

Impact: Once the concept is proven, additional carriers can be custom-designed, programmed, and developed to deliver therapeutic genes to various sub-populations of target breast cancer cells. In this research a 13aa breast cancer targeting peptide has been utilized as a targeting motif, but in future work other targeting motifs could be utilized including antibodies, growth factors, or aptamers specific to receptors expressed on the surface of breast cancer cells.

Nanomedicine and Drug Delivery Systems-2008

Engineering Biomimetic Vectors with Chimeric Architecture for Targeted Gene Therapy

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INTRODUCTION: The target sites for nucleic acid molecules used in gene therapy are mostly in the cell nucleus. Therefore, for vectors to be maximally effective, they should: 1) condense pDNA in order to protect them from serum endonucleases, 2) be recognized and internalized by target cells, 3) promote escape from the endosomal compartment, and 4) direct the nucleic acids towards the nucleus. *The objective* of this research is to genetically engineer a biomimetic vector that is able to condense pDNA into nano-size particles, target cancer cells specifically, disrupt endosomes, translocate pDNA towards nucleus, and transfect. **METHODS:** The vector was synthesized in BL21 (DE3) *E. coli* expression system and purified. The vector was complexed with pEGFP to form nanoparticles and characterized in terms of particle size and shape using a particle sizer and Transmission Electron Microscope (TEM), respectively. The nanoparticles were used to transfect breast cancer (ZR-75-1) and normal mammary cells (MCF-10A) in the presence and absence of chloroquine, bafilomycin, and nocodazole. The transfection efficiency was measured by flowcytometry. The ability of vector to target cancer cells was examined by an inhibition assay. The toxicity of vector was also studied using WST-1 assay. **RESULTS:** The gene carrier was synthesized with >98% purity. The average size of the nanoparticles was determined to be 68 ± 9nm at N/P ratio of 10 and with spherical shape. The vector/pEGFP complexes were able to target cancer cells specifically while showing no binding to normal mammary cells. The gene carrier was able to disrupt endosome membranes by fusogenic activity and proton sponge effect and translocate pDNA towards the nucleus via microtubules. **CONCLUSION:** This study demonstrates that by using component integration approach a multifunctional gene carrier can be engineered that is able to mimic virus characteristics and transfer genetic materials specifically to the target cancer cells.

Controlled Release Society 35th annual meeting, New York, NY, USA, Abstract #885

Development of a Recombinant Non-Viral Vector for Targeted Breast Cancer Gene Therapy

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msrsastry@wsu.com

ABSTRACT SUMMARY:

A multifunctional non-viral vector with chimeric architecture was engineered for selective targeting of human breast cancer cells. The vector was successfully tested for its comprehensive capability to condense plasmid DNA into nano-particles, specialized guided-delivery to cancer cells, disruption of endosomes, and efficient gene transfer.

INTRODUCTION:

Gene-transfer technology is still in a nacent stage owing to several inherent limitations. What has been long sought after is a technology with biocompatibility of ploxplexes and engineer-ability and efficiency of viruses in developing effective gene-transfer technology. An alternative biomimetic approach to non-viral gene-delivery system is at horizon but not without its own package of challenges to be overcome before making real advances in developing a feasible technology. A significant amount of preliminary groundwork has been done addressing the feasibility concerns of such an approach but more work needs to be done in this direction. Herein, we are reporting a component integration approach, wherein, several subsystems of diverse biological origin were integrated on modular platform to carry diverse functions of an efficient gene delivery vehicle. The resulting self-assembled, non-replicative and guided gene delivery module is designed to encompass the strengths of polyplexes and viral gene delivery vectors.

In an effort to develop a comprehensive non-viral targeted delivery vector, we have designed and engineered a multi-domain chimera protein. Using a biomimetic approach, the functional propensity of the concept protein was scripted into the primary sequence structure so as to carry an inherent information-base needed to perform an array of self-guided functions including: a) efficient condensation of the plasmid DNA into deliverable nano-particles, b) targeted delivery of the nano-particles using a combinatorially screened peptide [1] to target breast cancer cells, c) endosomal disruption using a fusogenic peptide facilitating escape of cargo into cytosol, and finally d) localization of the gene in the nucleus for gene expression. Because the vector is designed to target and deliver genes into breast cancer cells, it is named Breast Cancer Targeted Vector (BCTV).

EXPERIMENTAL METHODS:

Synthesis of adenovirus μ-peptide: A 19 amino acid adenoviral core peptide μ (mu) known for its efficient DNA neutralization and condensation [2] was synthesized by solid phase peptide synthesis.

Cloning of Breast Cancer Targeted Vector (BCTV): The pZERO resident synthetic BCTV gene flanked on either side by *NcoI* and *XhoI* restriction sites was cloned into pET21b vector under T7 promoter. The resultant expression construct pET21b:BCTV was tested for its fidelity to the original design by DNA sequencing.

Expression, purification, and identification of BCTV: pET21b:BCTV vector was transformed into *E. coli* BL21(DE3) pLysS. Transformants were grown in Circlegrow media and induced with IPTG for 2 hours. The expressed protein vector was purified to homogeneity on a Ni-NTA column. The purity and expression of the protein vector were determined by SDS-PAGE and western blot analysis, respectively.

Particle characterization: Vector/pDNA nano-complexes were prepared at various stoichiometric N/P ratios (the molar ratio of nitrogen atoms of vector to phosphates in pDNA) by adding various amounts of the μ peptide or fusion-protein into 3 μg of pDNA (pEGFP). The mean hydrodynamic size of vector/pDNA complexes was measured by Photon Correlation Spectroscopy (PCS).

Gel retardation assay: Completion of DNA condensation was established empirically for the theoretically formed stoichiometric nano-complexes employing gel retardation assay on a 0.7% agarose gel visualized by ethidium bromide staining. The amount of protein used at this ratio, hence, was used as an equimolar charge neutralization factor of 1.

Cell culture and transfection: ZR-75-1 breast cancer and MCF-10A normal human mammary cells were seeded in 12 well tissue culture plates. Cells were approximately 80% confluent at the time of transfection. pEGFP was mixed with various ratios of μ peptide or BCTV vector for complex formation followed by addition to the growth media. When used, chloroquine (100μM) was added to assist the

particles escape into cytoplasm. The expression of GFP was visualized after 48 hrs by epifluorescence microscopy and the percent transfected cells was determined by flow cytometry.

RESULTS AND DISCUSSION:

BCTV expression, purification, and identification: A homogenous band consistent with the molecular weight of BCTV at 11.4 kDa on SDS-PAGE was positively identified by western blot analysis (**Figure 1**).

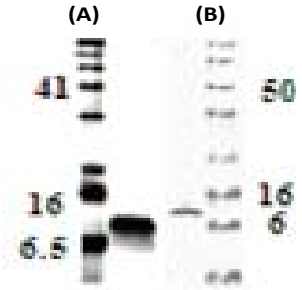

Figure 1: SDS-PAGE and western blot analysis of purified BCTV. **A)** SDS-PAGE of purified BCTV with >98% purity. **B)** Western blot analysis of purified BCTV using anti-his tag antibody.

Particle size analysis and gel retardation assay: PCS analysis of BCTV/pDNA complexes demonstrated a stable particle size of ca. 80 nm over a wide empirical N/P ratio. It was observed that at N/P ratio 2:1 the pDNA was fully neutralized and the size of the particles at this ratio was ca. 70 nm. We did not observe any appreciable size change even at higher ratios of 12:1. However, for μ peptide/ pDNA complexes, the average size at empirical neutralization value of 4:1 was ca. 150 nm. Higher N/P ratios resulted in larger particle sizes and high polydispersity index. We speculate that the neutralization of the surface charge resulted in the aggregation of particles. A dose dependent decrease in the migration pattern for both vectors on a 0.7% agarose gel indicated the vector/pDNA interactions **(Figure 2)**. At N/P ratios below 2, portion of pDNA exists as un-neutralized with a high polydispersion index (PDI).

Cell transfection studies: In cell transfection studies, pEGFP encoding Green Fluorescent Protein was condensed with either BCTV or μ peptide at different N/P ratios and used to transfect ZR-75-1 carcinoma breast cell line and MCF-10A normal epithelial mammary cells.

Cell transfection was performed in the absence and presence of chloroquine to evaluate the efficacy of endosomal disruption motif. Vector without targeting peptide, namely μ peptide, was used to study the effect of targeting peptide in enhancing particle uptake.

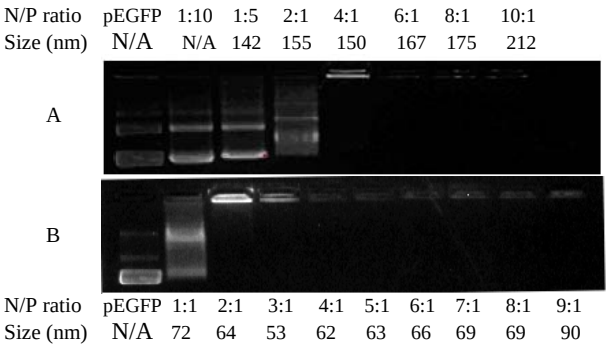

Figure 2: Agarose gel electrophoresis of A) μ peptide and B) BCTV complexes with pDNA at various N/P ratios and corresponding particle size analysis.

In the absence of chloroquine, μ peptide was unable to transfect the ZR-75-1 or MCF-10A Cell lines. In the presence of chloroquine in both cell lines, the transfection levels were below 1% and comparable indicating non-specific uptake.

In ZR-75 cells, the transfection efficiency of BCTV was nine fold higher (900% increase) than μ peptide. This indicates that presence of TP in the BCTV facilitated internalization of nanoparticles and resulted in higher transfection efficiency.

In the absence of chloroquine, the transfection efficiency of BCTV in ZR-75 was ninety times higher than μ peptide, demonstrating the ability of the endosome disrupting motif in BCTV structure to enhance transfection efficiency.

CONCLUSION:

We conclude that aided by the DNA condensing peptide μ, the combinatorially screened targeting peptide TP, and fusogenic peptide, the BCTV is an efficient multifunctional delivery module capable of condensing the pDNA into deliverable nano-particles and exhibits specialized targeting of breast cancer cells. Preliminary results being encouraging, we are in a process of further optimization to enhance the basal level of transfection efficiency and increase the endosomal escape.

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